Please add claim 19 as follows:

Cont,

19. The method of claim 1, wherein said antigen presenting cell express high level of Fas ligand generated by adenovirus AdLoxPFasL and AxCANCre.

REMARKS

The 35 U.S.C. §102(e) Rejection

Claim 17 stands rejected under 35 U.S.C. §102(e) as being anticipated by **Bellgrau** et al.

Claim 17 has been cancelled. Accordingly, this rejection is now moot.

The 35 U.S.C. §103(a) Rejection

Claims 1, 3-6 and 17 stand rejected under 35 U.S.C. §103(a) as being unpatentable over **Bellgrau** et al. in view of **Süss** et al. This rejection is respectfully traversed.

Bellgrau et al. teach a method of inhibiting T-lymphocyte-mediated immune responses by providing a recipient animal with Fas ligand or cells expressing Fas ligand. **Bellgrau** et al.

do not teach the use of antigen presenting cells to express Fas ligand in said method. **Süss** et al. teach that a sub-population of dendritic cells ($CD8^+$ dendritic cells) express Fas ligand and induce apoptosis of $CD4^+$ T cells which results in the down regulation of the immune response. However, a person having ordinary skill in the art at the time the invention was made would not have a reasonable expectation of successfully producing Applicants' claimed invention by simply combining the teaching of **Bellgrau** et al. and **Süss** et al.

High expression of Fas ligand can lead to immune cell infiltration and inflammation instead of immunosuppression. For example, **Kang** et al. report that Fas ligand expression on pancreatic islets results in neutrophilic infiltration and accelerated graft rejection (enclosed). **Chen** et al. report subcutaneous injection of stably transfected colon carcinoma cells that express Fas ligand results in neutrophils activation and rejection of the cancer cells (enclosed). Hence, expression of Fas ligand does not always inhibit immune responses. The fact that Fas ligand expression can lead to enhancement as well as inhibition of immune responses indicates that the regulatory function of Fas ligand is more complex and varies between different experimental and in vivo settings.

The effect of Fas ligand expression on an immune response also depends on the presence or absence of other

regulatory factors in that particular site (**Chen** et al., page 1715, left column, lines 17-20). However, **Bellgrau** et al. only show results with soluble Fas ligand and Fas ligand expressed on islet cells, which are not antigen presenting cells. **Süss** et al. only show data from in vitro culture. It is well known to one in the art that one cannot equate in vitro data to in vivo results. The differences between in vitro and in vivo situations becomes more important as **Chen** et al. teach that the in vivo microenvironment and the presence of secondary factor play an important role in regulating the effect of Fas ligand expression (see abstract; page 1715, left column, lines 17-20; page 1715, middle column, lines 1-4). Therefore, the combined teaching of **Bellgrau** et al. and **Süss** et al. does not address the potential problem of inflammation induced by Fas ligand expression, and the data in the combined references do not necessarily lead to the conclusion that Fas ligand expression on antigen presenting cells would lead to immunosuppression in transplantation or autoimmunity in vivo.

Moreover, there are aspects of Applicants' claimed invention which are neither taught nor suggested in the combined teaching of **Bellgrau** et al. and **Süss** et al. Applicants' claimed invention uses Fas negative antigen presenting cells derived from the mutant *lpr/lpr* mice to present specific antigen and the Fas ligand

death signal to naive T cells. Previous investigators have used Fas ligand therapy in Fas positive cells. High levels of Fas ligand expression in Fas positive cells can lead to the killing of those Fas ligand expressing cells. By using Fas negative antigen presenting cells, high level of Fas ligand expression and cell survival can be achieved and maintained. Applicants' claimed invention also uses a two virus system, adenovirus AdLoxPFasL combined with the second adenovirus AxCANCre, to achieve a very high level of Fas ligand expression. The use of this adenovirus system generates almost 100-fold higher Fas ligand expression compared to conventional transfection (Example 19, Fig. 9). These features of Applicants' invention are recited in the amended claims. The use of Fas negative antigen presenting cells that can express a very high level of Fas ligand is not taught nor suggested in the teaching of **Bellgrau** et al. and **Süss** et al.

The present invention expresses Fas ligand on antigen presenting cells which can be used as universal cells to present antigens, activate and specifically kill T cells. Injected antigen presenting cells migrate to the spleen, thus Fas ligand is targeted primarily to the spleen via antigen presenting cells and very seldom to other sites. This puts Fas ligand in the precise place where it induces a maximum benefit towards interaction with autoreactive T

cells, resulting in systemic and not local tolerance. In contrast, **Bellgrau**'s Fas ligand cannot be specifically targeted to the spleen but might migrate to the liver and other tissues and cause high toxicity. Therefore, the combined teaching of **Bellgrau** et al. and **Süss** et al. do not encompass all the important aspects of Applicants' claimed invention.

In view of the above remarks, **Bellgrau** et al. in combination with **Süss** et al. do not provide a person having ordinary skill in this art with the requisite motivation nor expectation of successfully producing Applicants' claimed methods. The invention as a whole is not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the cited references. Accordingly, Applicants respectfully submit that the rejections of claims 1, 3-6 and 17 under 35 U.S.C. §103(a) be withdrawn.

Claims 1, 3-6 and 17 stand rejected under 35 U.S.C. §103(a) as being unpatentable over **Bellgrau** et al. in view of **Schuler** et al. This rejection is respectfully traversed.

Bellgrau et al. teach a method of inhibiting T-lymphocyte-mediated immune responses by providing a recipient

animal with Fas ligand or cells expressing Fas ligand. **Schuler** et al. is a review article which discusses dendritic cell expression of Fas ligand and this approach to induce tolerance in transplantation and autoimmunity. However, a person having ordinary skill in the art at the time the invention was made would not have a reasonable expection of successfully producing Applicants' claimed invention by simply combining the teaching of **Bellgrau** et al. and **Schuler** et al. Fas ligand expression does not always lead to immune response suppression. High expression of Fas ligand can lead to immune cell infiltration and inflammation instead of immunosuppression as reported in **Kang** et al. and **Chen** et al. Therefore, Fas ligand expression can lead to enhancement as well as inhibition of immune response. The effect of Fas ligand expression on an immune response depends on a number of other factors such as the level of Fas ligand expression (**Kang** et al., page 742, left column, lines 45-59), the specific microenvironment and the presence of other regulatory factor (**Chen** et al., page 1715, left column, lines 17-20; page 1715, middle column, lines 1-4). However, **Bellgrau** et al. only show results with soluble Fas ligand and Fas ligand expressed on islet cells, which are not antigen presenting cells. Although **Schuler** et al. suggest that dendritic cells express Fas ligand and may provide a novel approach to induce tolerance in transplantation and

autoimmunity, they do not have any data to show Fas ligand expression on antigen presenting cells would has inhibitory rather than stimulatory effect in vivo. Moreover, this suggestion in **Schuler** et al. is an oversimplified statement in view of the teaching in **Kang** et al. **Kang** et al. report that they "have found in a clinically relevant transplant model that FasL acts as proinflammatory factor that accelerates, rather than prevents graft loss. This proinflammatory function of FasL will require further characterization and modulation before FasL-based immunotherapies can be applied" (page 742, right column, lines 16-20). Hence, the combined teaching of **Bellgrau** et al. and **Schuler** et al. neither address the potential problem of inflammation induced by Fas ligand expression, nor provide sufficient data for the conclusion of successful inhibition of immune response by expressing Fas ligand on an antigen presenting cell in vivo.

Moreover, there are aspects of Applicants' claimed invention which are neither taught nor suggested in the combined teaching of **Bellgrau** et al. and **Schuler** et al. As discussed above, the use of Fas negative antigen presenting cells that can express a very high level of Fas ligand as described in Applicants' claimed invention is not taught nor suggested in the teaching of **Bellgrau** et al. and **Schuler** et al. The advantages of using antigen presenting

cells that can carry Fas ligand expression to the spleen for maximal interaction with T cells and generate minimal toxicity are not taught nor suggested in the teaching of **Bellgrau** et al. and **Schuler** et al. Therefore, the combined teaching of **Bellgrau** et al. and **Schuler** et al. does not encompass all the aspects of Applicants' claimed invention.

In view of the above remarks, **Bellgrau** et al. in combination with **Schuler** et al. do not provide a person having ordinary skill in this art with the requisite motivation nor expectation of successfully producing Applicants' claimed methods. The invention as a whole is not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by contrary references. Accordingly, Applicants respectfully submit that the rejections of claims 1, 3-6 and 17 under 35 U.S.C. §103(a) be withdrawn.

Claims 16 stand rejected under 35 U.S.C. §103(a) as being unpatentable over **Bellgrau** et al. in view of **Süss** et al., as well as unpatentable over **Bellgrau** et al. in view of **Schuler** et al. The rejection is respectfully traversed.

As discussed above, the combined teaching of **Bellgrau** et al. and **Süss** et al., as well as that of **Bellgrau** et al. and **Schuler**

et al. do not provide one of ordinary skill in the art at the time the invention was made a reasonable expectation of success in producing the claimed invention because of the potential problem of inducing inflammation and enhancing immune response by Fas ligand expression. The combined references do not have any data on the effect of Fas ligand expression on antigen presenting cell in vivo. In the absence of the necessary teaching or suggestion that Fas ligand expression in the in vivo situations described in Applicants' claimed invention would have inhibitory rather than stimulatory effects, the combined teaching will not lead one of ordinary skill in the art to the claimed invention. Moreover, the important features of using Fas negative antigen presenting cells and inducing high level of Fas ligand expression by a novel adenovirus system are not taught or suggested in the combined references. Based on the above remarks, **Bellgrau** et al. in view of **Süss** et al. or in view of **Schuler** et al. do not render claim 16 obvious. Accordingly, Applicants respectfully request that the rejection of claim 16 under 35 U.S.C. §103(a) be withdrawn.

This is intended to be a complete response to the Office Action mailed October 29, 1999. Applicants submit that the pending

claims are in condition for allowance. If any issues remain, please telephone the attorney of record for immediate resolution.

Respectfully submitted,

Date: Dec 3, 1999

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experienced an "extra" duplication event. This suggests that a fish-specific *HOX* cluster duplication occurred before the divergence of *Fugu* and zebrafish lineages more than 150 million years ago (*15*), but after the divergence of ray-finned and lobe-finned lineages. Goldfish, salmonids, and some other teleosts have experienced additional, more recent polyploidization events (*16*). Genomic analysis of basally branching ray-finned fish, such as sturgeons, *Amia*, or *Polypterus*, is necessary to clarify the timing of the *HOX* duplication event.

To determine whether "extra" fish *hox* clusters result from tandem duplication or chromosome duplication in fish, or cluster loss in tetrapods, we mapped zebrafish *hox* clusters: cloned, sequenced, and mapped four new genes whose orthologs are syntenic with *HOX* clusters in mammals (*dhh*, *evx1*, *ang1b*, and *gli*); and mapped four previously unmapped zebrafish genes [*dlx5*, *dlx6*, *dlx8*, and *pl10a*; see (*11*)] whose orthologs are linked to *HOX* clusters in mammals. These experiments showed that zebrafish has two copies of each *HOX* chromosome segment in mammals (Fig. 4). For example, the human and mouse *HOXB* chromosomes have six and four genes, respectively, whose apparent orthologs map on one of the two zebrafish chromosomes containing *hoxba* or *hoxbb* (Fig. 4). Each of these two chromosomes also has one copy of other duplicate genes, including *dlx7/dlx8*, *rara2a/rara2b*, and *hbae4/hbae1* (*11*, *17*). We conclude that zebrafish has two copies of this mammalian chromosome segment. Because similar results were obtained for the other clusters (Fig. 4), we infer that *hox* cluster duplication in ray-finned fish occurred by whole chromosome duplication. Although we found a single *hoxd* cluster in zebrafish, mapping experiments identified the predicted duplicate chromosome segments (Fig. 4), suggesting secondary loss of one *hoxd* duplicate.

These results suggest two rounds of *HOX* chromosome duplication (probably whole genome duplication) before the divergence of ray-finned and lobe-finned fishes, and one more in ray-finned fish before the teleost radiation. Because gene duplicates often have a subset of the functions of the ancestral gene (*18*), mutations in duplicate genes may reveal essential functions that otherwise might remain hidden. For example, if a gene is essential for distinct early and late functions, a lethal mutation knocking out the early function might obscure the late function in a mutant mammal, but both functions would be evident if the two functions assort to different zebrafish gene duplicates. The conclusion that the genetic complexity of *hox* clusters in teleost fish has exceeded that of mammals for more than 100 million years calls into question the concept of a tight linkage of *HOX* cluster number and morphological complexity along the body axis. However, because teleosts are the most species-rich group of vertebrates and exhibit tremendous morphological diversity, it is tempting to speculate that the duplication event detected here may have provided gene copies that helped spur the teleost radiation.

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9. DNA pools (plate pools, then row and column pools) from an arrayed zebrafish PAC library (*19*) were screened with redundant primers (Hox-F: 5′-CGNAAGAARMGNGTNGGNTAYAC-3′; Hox-R: 5′-GATNCKNGKRTTYTGRAACCANAT-3′; Hox-Fi: 5′-GTNGARYTNGARAARGARTTYGA), where N is A, C, T, or G, R is A or G, M is A or C, Y is C or T, and K is T or G. Ends of positive PACs were sequenced and specific primers used to find overlapping clones. Positive PACs were amplified with redundant primers; products were cloned and sequenced, and gene specific primers were used to obtain sequence directly from PAC DNA.
10. Unambiguously alignable sequences were obtained using CLUSTAL X (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and trees were generated by the neighbor-joining method [N. Saitou and M. Nei, *Mol. Biol. Evol.* **4**, 406 (1987)]. A lamprey (*Petromyzon marinus*) cDNA library screened with redundant *hox* gene primers provided an outgroup. For accession numbers, see (*11*).
11. See *Science* Online (www.sciencemag.org) for a catalog of zebrafish *hox* genes, alignments for the phylogenetic trees in Fig. 2, accession numbers of loci used to construct the phylogenetic trees, mapping primers for zebrafish genes, and mapping data for newly mapped loci.
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18 June 1998; accepted 23 October 1998

Regulation of the Proinflammatory Effects of Fas Ligand (CD95L)

Jian-Jun Chen, Yongnian Sun, Gary J. Nabel*

Fas ligand (CD95L) inhibits T cell function in immune-privileged organs such as the eye and testis, yet in most tissues CD95L expression induces potent inflammatory responses. With a stably transfected colon carcinoma cell line, CT26-CD95L, the molecular basis for these divergent responses was defined. When injected subcutaneously, rejection of CT26-CD95L was caused by neutrophils activated by CD95L. CT26-CD95L survived in the intraocular space because of the presence of transforming growth factor–β (TGF-β), which inhibited neutrophil activation. Providing TGF-β to subcutaneous sites protected against tumor rejection. Thus, these cytokines together generate a microenvironment that promotes immunologic tolerance, which may aid in the amelioration of allograft rejection.

The CD95 protein (also called Fas or APO-1) is a cell surface receptor that activates the death signaling pathway in cells. Its physiological ligand, CD95L, can transduce this signal upon cell contact (*1*). The CD95-CD95L system has been implicated in the clonal deletion of autoreactive lymphocytes in peripheral lymphoid tissues and in the elimination of autoreactive lymphocyte populations (*2*), thus contributing to homeostasis

of the immune system. CD95L expression in normal tissue is restricted to T lymphocytes, macrophages, the cornea, the iris, ciliary bodies, the retina, and Sertoli cells (*3*). Through its ability to suppress both cellular and humoral immunity (*2*, *4*), CD95L has been implicated in maintenance of the immune-privileged status in the eye (*3*) and testis (*5*). CD95L may also confer immune suppression in malignancy (*6*) or be useful in delaying rejection of allogeneic cells (*4*, *7*) by promoting immune evasion.

However, CD95L is also proinflammatory (*8*, *9*). Expression of CD95L on myotubes or pancreatic islets of transgenic mice induces a granulocytic response that accelerates graft rejection (*10*). Differences in the effect of CD95L at distinct anatomic sites may be caused by secondary factors that modulate its function (*11*). Here we attempt to identify factors that could explain the paradoxical effects of CD95L in immune-privileged sites and immunocompetent tissues.

To determine whether CD95L could stimulate an inflammatory response in an immune-privileged tissue, we injected 10^5 CT26-CD95L cells (*4*) (n = 5 mice) or a CD95L-negative control cell line, CT26-Neo (n = 4 mice), into the anterior chamber of the eye of syngeneic Balb/c mice. Both cell lines produced tumors by 8 days in all mice at the intraocular site. In contrast, tumors grew only from CT26-Neo cells when the tumor cells were injected subcutaneously (*12*). Thus, the microenvironment, rather than the amount of CD95L, determined the ability of these cells to induce inflammatory responses that inhibited tumor growth.

Rejection of CD95L$^-$ tumors occurs in *scid-beige* mice and is thus independent of T cell and natural killer (NK) cell function (*6*). Polymorphonuclear leukocytes (PMNs) infil-

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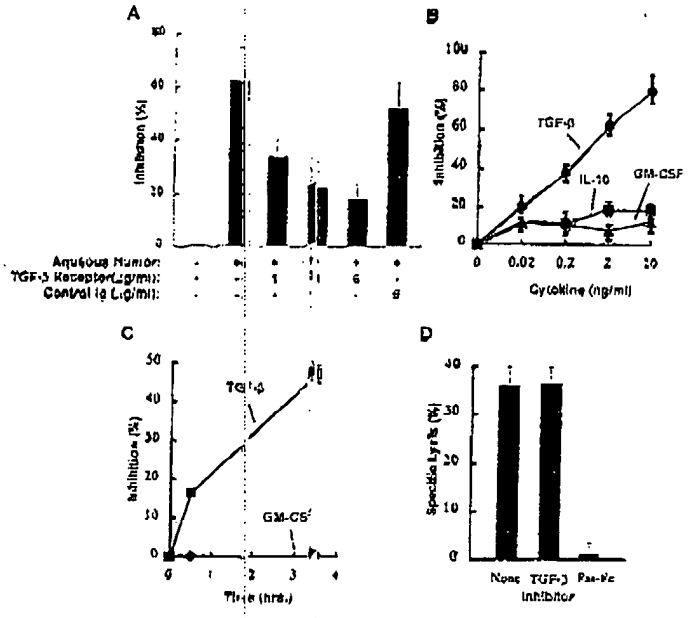

Fig. 2. Inhibitory effects of recombinant TGF-β on neutrophil-mediated cytotoxicity and its role in the aqueous humor. (**A**) Inhibitory effects of aqueous humor and reversal by neutralizing soluble TGF-β receptor protein. Mouse neutrophils were incubated with radiolabeled CT26-CD95L at a ratio of 50:1 and 40 μl of bovine aqueous humor, obtained immediately after the animal was killed and stored at −70°C. The indicated concentrations of human TGF-β–soluble receptor-Fc fusion protein (TGF-β SRII/Fc; R&D Systems) or control human immunoglobulin were added to the assay as shown in Fig. 1C. The specific lysis without inhibitor was 44.3%. (**B**) Inhibition of neutrophil cytolysis by TGF-β. Human PMNs were incubated with CT26-CD95L cells at an E/T ratio of 100:1. Increasing amounts of human TGF-β1 (R&D Systems), human interleukin-10 (Genzyme), and granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex) were added to the culture, and Cr release assays were performed as in Fig. 1. The specific lysis without inhibitors was 44.3%. (**C**) Inhibition of neutrophil-mediated cytolysis of CT26-CD95L cells by preincubation of neutrophils with TGF-β1. Human PMNs were preincubated with TGF-β1 (20 ng/ml) or human GM-CSF (20 ng/ml) for the indicated period and washed with 10 ml of medium three times. The percent inhibition was calculated relative to neutrophils preincubated with medium, TGF-β1, or GM-CSF for the same period of time. The data represent the mean ± SE from three independent experiments. (**D**) TGF-β1 does not inhibit CD95L-mediated apoptosis in Jurkat cells. CT26-CD95L cells were incubated with ^{51}Cr-labeled Jurkat cells (1×10^4 per well) at a ratio of 10:1. Human TGF-β1 (20 ng/ml) was added to the medium, and CD95-Fc fusion protein was used as a positive control. Cytotoxicity was measured by ^{51}Cr release after 4 hours of coincubation. The data represent the mean ± SD from three independent experiments.

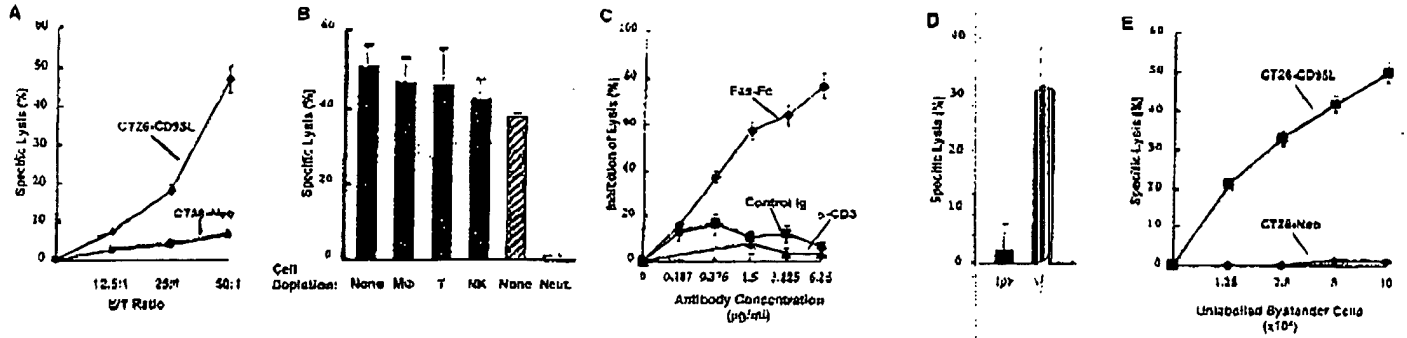

Fig. 1. PMN-mediated destruction of CD95L$^+$ CT26 cells but not CD95L$^-$ CT26 cells in vitro and CD95L involvement in neutrophil cytolytic function. (**A**) Dose-dependent cytolysis of CD95L$^+$ CT26 cells by human neutrophils. Human neutrophils (*20*) of ≥97% purity were incubated with ^{51}Cr-labeled CT26-CD95L or CT26-Neo cells for 19 hours on fibronectin-coated plates (*21*) at the indicated ratios. The data represent the mean ± SE of three independent experiments. (**B**) PMN-mediated cytolysis. Human PMNs (black bars) were depleted of T lymphocytes, NK cells, or macrophages by immunomagnetic bead separation (*22*) with antibodies to CD3, CD65, or CD115/c (Neomarkers: Pharmingen) and were mixed with CD95L$^+$ cells (50:1 ratio). Mouse neutrophils (gray bars) (*23*) were mixed with CD95L$^-$ cells (50:1 ratio). Mouse spleen cells depleted of neutrophils with antibody to Ly6G (Pharmingen) (*22*) were used as a negative control. (**C**) Inhibition of neutrophil cytotoxicity by CD95-Fc (*4*). Radiolabeled CT26-CD95L cells were mixed with neutrophils [effector/target (E/T) ratio, 50:1], and mouse CD95-Fc protein, control human immunoglobulin, or antibody to CD3 (OKT3, Ortho Biotech) was added. The specific lysis without inhibitor was 37.5%. (**D**) Lysis of CT26-CD95L by neutrophils from *lpr*$^-$/*lpr*$^-$ or wild-type congenic C57BL/6 mice at an E/T ratio of 25:1. (**E**) Induction of bystander cytotoxicity by CT26-CD95L cells. CT26-Neo target cells were labeled with ^{51}Cr and mixed with neutrophils at an E/T ratio of 100:1 in the presence of the indicated numbers of unlabeled CT26-CD95L cells. Equal numbers of unlabeled CT26-Neo cells were used as a negative control.

trate CD95L tumors in the skin and contribute to the CD95L antitumor response (*8, 9*), but the mechanism by which these cells promote tumor rejection is unknown. We therefore determined whether neutrophils could directly lyse CT26-CD95L cells in vitro. Human PMNs, incubated with CT26-CD95L or CT26-Neo target cells, lysed only CT26-CD95L (Fig. 1A). Depletion of the effector population with antibodies to T cells, NK cells, or macrophages did not reduce this cytolysis. PMNs derived from peripheral blood leukocytes of syngeneic Balb/c mice lysed CT26-CD95L tumor cells, in contrast to a control cell population depleted with a neutrophil-specific antibody, which did not retain this activity (Fig. 1B). Thus, PMNs interacted directly with CD95L$^+$ cells to mediate their destruction.

Lysis of CT26-CD95L cells was inhibited specifically by a CD95-Fc fusion protein but not by a negative control immunoglobulin (Fig. 1C) and was markedly reduced in neutrophils from *lpr* mice (Fig. 1D), which express a defective CD95 receptor. Bystander cells that did not express CD95L were lysed when chromium-labeled CT26-neo cells were incubated with unlabeled CT26-CD95L cells (Fig. 1E), which suggests that lysis of CT26-CD95L cells was not due to their intrinsic susceptibility to lysis but instead to the ability of CD95L to induce neutrophil cytotoxicity locally.

Tumors can grow intraocularly but not in subcutaneous sites. The microenvironment of the eye may contain factors, therefore, that suppress PMN activation. We tested the fluid of the anterior chamber (the aqueous humor) in cytotoxicity assays and found that it inhibited CD95L activation of PMN lytic activity (Fig. 2A). We tested several cytokines known to be present in the aqueous humor (*13*). Transforming growth factor-β1 (TGF-β1) inhibited PMN cytotoxicity in vitro (Fig. 2B), and a soluble TGF-β receptor-Fc fusion protein inhibited the suppressive effect of aqueous humor in vitro (Fig. 2A). The effect of TGF-β was on the PMNs (Fig. 2C), and the same dose of TGF-β1 had no effect on CD95L-dependent apoptosis of Jurkat cells (Fig. 2D). A similar inhibitory effect on neutrophil function was observed with human TGF-β2 (*14*).

To understand the mechanism of PMN inhibition further, we examined the activity of mitogen-activated protein kinase (MAPK) in CD95-stimulated human neutrophils. Rapid activation of p38 MAPK activity, as determined on its substrate, ATF-2 (*15*), was demonstrated in CD95L-stimulated neutrophils (Fig. 3A, lane 2). Preincubation of neutrophils with TGF-β1 suppressed this CD95L-induced activation of p38 MAPK (Fig. 3A, lane 3). Similarly to TGF-β, incubation of neutrophils with the p38 MAPK inhibitors SB203580 and SB202190 reduced neutrophil-mediated cytolysis, in contrast to a p44/42 antagonist, PD98059, that had no effect (Fig. 3B). These results demonstrated that CD95L-induced neutrophil cytotoxicity was dependent on p38 MAPK function, which is inhibited by TGF-β.

To determine whether expression of TGF-β could affect the proinflammatory effect of CD95L in subcutaneous tissue, CT26-CD95L cells were stably transfected with an expression vector encoding a constitutively active form of TGF-β1. Histologic analysis confirmed fibrosis and regression of CT26-CD95L, as reported previously (*8*), in contrast to the robust tumor growth of the double transfectants (Fig. 4A, upper panel). Occasional neutrophils were observed in the TGF-β–expressing tumors and in intraocular CT26-CD95L (Fig. 4A, lower panel), which suggests that TGF-β suppressed CD95L-induced PMN activation, although an effect on migration or survival in vivo cannot be excluded. All CT26-CD95L/TGF-β cells (n = 8) grew in recipient mice when inoculated subcutaneously, in contrast to no growth in any recipient of CT26-CD95L cells (n = 8) (Fig. 4B).

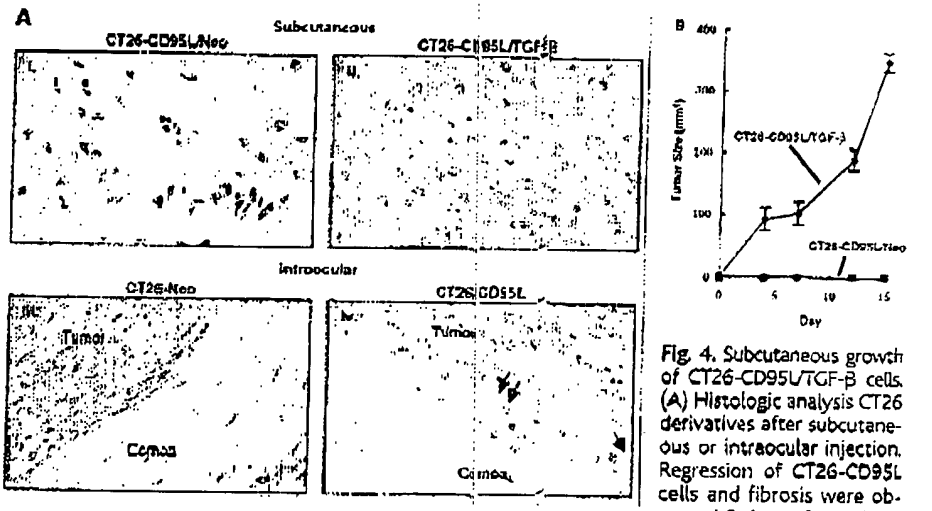

Fig. 4. Subcutaneous growth of CT26-CD95L/TGF-β cells. (A) Histologic analysis CT26 derivatives after subcutaneous or intraocular injection. Regression of CT26-CD95L cells and fibrosis were observed 8 days after subcutaneous inoculation (*i*), in contrast to the observation of viable tumor cells after injection with CT26-CD95L/TGF-β cells (*ii*). Tumor growth was observed intraocularly for both CT26-Neo (iii) and CT26-CD95L (iv) tumors. Arrows indicate the presence of occasional neutrophils in CT26-CD95L tumors intraocularly (iv). (B) Mice were injected subcutaneously with CT26-CD95L cells (n = 8) or with a subline stably transduced with a retroviral vector encoding constitutively active TGF-β1 (n = 8). Tumor volume, measured with calipers, was calculated from the largest (*a*) and smallest (*b*) diameter with the formula $0.5a \times b^2$. CT26-CD95L/TGF-β cells were generated by infection in CT26-CD95L cell line with a retrovirus vector (24). A constitutively active human TGF-β1 gene (25) was cloned into retroviral vector LZRS-pBMN-lacZ (25) by replacing the *lacZ* gene. TGF-β1 levels in supernatants 48 hours after infection were 8 ng/ml of TGF-β1, determined by enzyme-linked immunosorbent assay (R&D Systems). CT26-CD95L cells infected with control retroviruses (CT26-CD95L/Neo) were used as controls.

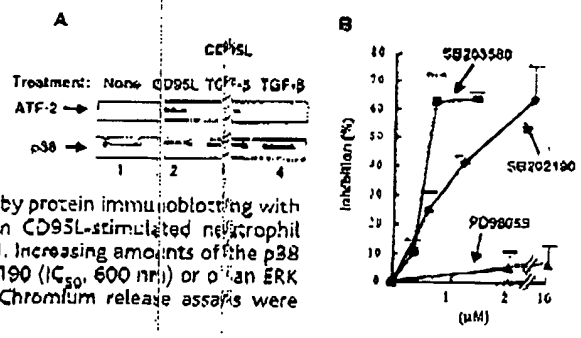

Fig. 3. p38 MAPK activation by CD95L, inhibition by TGF-β, and abrogation of CD95L-induced neutrophil cytolysis by p38 MAPK antagonists. (A) Activation and modulation of p38 MAPK in neutrophils by CD95L and inhibition by TGF-β. (Upper panel) Cellular p38 MAPK activity was determined by phosphorylation of an ATF2 substrate (New England Biolabs) after immunoprecipitation. Neutrophils were pretreated with human TGF-β1 (20 ng/ml, lanes 3 and 4) or with medium (lanes 1 and 2) at 4°C for 1 hour. Subsequently, the neutrophils were cocultured with human CD95L (400 ng/ml) (Upstate Biotechnology; lanes 2 and 3) or with medium (lanes 1 and 4) for 10 min at 37°C. (Lower panel) The total amount of p38 MAPK was examined by protein immunoblotting with p38 kinase antibody (New England Biolabs). (B) Effects of p38 MAPK inhibitors on CD95L-stimulated neutrophil cytotoxicity. Neutrophils were incubated with CT26-CD95L cells at an E/T ratio of 50:1. Increasing amounts of the p38 MAPK inhibitors SB203580 (median inhibitory concentration (IC_{50}), 350 nm) or SB202190 (IC_{50}, 600 nm) or of an ERK kinase inhibitor (negative control), PD98059 (IC_{50}, 2 μm), were added to media. Chromium release assays were performed as in Fig. 2. The specific lysis without inhibitor was 45%.

The proinflammatory effects of CD95L have raised questions about its contribution to immune privilege (*3, 5, 16*), tolerance, and graft survival (*8–10*). Although it triggers apoptosis in T lymphocytes (*2*) (Fig. 2D), CD95L unexpectedly stimulated PMN activation. As described for other PMN stimulants (*17*), this activity is dependent on its ability to enhance p38 MAPK activity (Fig. 3). PMNs directly mediate cytolysis of CD95L$^+$ cells, and this effect is inhibited by TGF-β, which is present in the aqueous humor (*13*). TGF-β also plays a role in immune tolerance through this mechanism and its effect on T cell proliferation. Although it inhibits p38 MAPK activity in other cells (*18*), its effect on innate immune responses mediated by neutrophils was previously unknown. Together CD95L and TGF-β promote lymphocyte clonal deletion and suppress inflammation. Thus, providing a microenvironment that includes both of these elements may aid in amelioration of allograft rejection at nonprivileged sites. Both CD95L and TGF-β1 have also been detected in tumors, particularly in the extracellular matrix, where they may inhibit immunologic recognition of malignancies (*6, 19*). Successful immune therapies for cancer are likely to require strategies to reverse this mechanism of immune suppression in vivo.

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Identification of Two Distinct Mechanisms of Phagocytosis Controlled by Different Rho GTPases

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The complement and immunoglobulin receptors are the major phagocytic receptors involved during infection. However, only immunoglobulin-dependent uptake results in a respiratory burst and an inflammatory response in macrophages. Rho guanosine triphosphatases (molecular switches that control the organization of the actin cytoskeleton) were found to be essential for both types of phagocytosis. Two distinct mechanisms of phagocytosis were identified: Type I, used by the immunoglobulin receptor, is mediated by Cdc42 and Rac, and type II, used by the complement receptor, is mediated by Rho. These results suggest a molecular basis for the different biological consequences that are associated with phagocytosis.

Phagocytosis is the process by which cells recognize and engulf large particles (>0.5 μm) and is important to host defense mechanisms as well as to tissue repair and morphogenetic remodeling. Two of the best characterized phagocytic receptors in macrophages, the complement receptor 3 (CR3) and Fc gamma receptors (FcγRs), are involved in the uptake of opsonized microorganisms during infection. CR3 binds C3bi on complement-opsonized targets, whereas FcγRs bind to immunoglobulin G (IgG)–coated targets. Phagocytosis by both types of receptors is driven by the reorganization of filamentous actin (F-actin), but the mechanisms of uptake appear to be different (*1, 2*). First, FcγR-mediated uptake is accompanied by pseudopod extension and membrane ruffling, whereas complement-opsonized targets sink into the cell, producing little protrusive activity (*3*). Second, FcγR ligation is accompanied by the activation of the respiratory burst (to produce reactive oxygen species) and by the production of arachidonic acid metabolites and cytokines, such as tumor necrosis factor–α. C3bi-dependent uptake occurs in the absence of any of these proinflammatory signals (*4–6*).

The Rho family of small guanosine triphosphatases (GTPases) is involved in the reorganization of filamentous actin structures in response to extracellular stimuli (*7*). Rho induces the assembly of contractile actomyosin filaments, whereas Rac and Cdc42 control actin polymerization into lamellipodial and filopodial membrane protrusions, respectively (*8, 9*). In addition, these GTPases can affect gene transcription [through the activation of nuclear factor kappa B, through the c-Jun NH_2-terminal kinase (JNK), and through the p38 mitogen-activated protein kinase (MAPK)], and Rac regulates the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex that is responsible for the respiratory burst (*10, 11*). We have, therefore, analyzed the relative roles of Rho, Rac, and Cdc42 in FcγR- and CR3-mediated phagocytosis.

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ARTICLES

Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction

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Fas ligand is believed to mediate immune privilege in a variety of tissues, including the eye, testis, and a subset of tumors. We tested whether expression of Fas ligand on pancreatic islets either following adenoviral or germline gene transfer could confer immune privilege after transplantation. Islets were infected with an adenoviral vector containing the murine Fas ligand cDNA (AdFasL), and were transplanted into allogenic diabetic hosts. Paradoxically, AdFasL-infected islets underwent accelerated neutrophilic rejection. The rejection was T cell and B cell independent and required Fas protein expression by host cells, but not on islets. Similarly, transgenic mice expressing Fas ligand in pancreatic β cells developed massive neutrophilic infiltrates and diabetes at a young age. Thus, Fas ligand expression on pancreatic islets results in neutrophilic infiltration and islet destruction. These results have important implications for the development of Fas ligand-based immunotherapies.

Fas ligand (FasL, CD95L) is a type II transmembrane protein of the tumor necrosis factor family that induces cells to send an apoptotic signal to cells expressing Fas (CD95, APO-1)[1,2]. FasL is expressed on a limited number of cell types, including activated T cells, Sertoli cells in the testis, and epithelial cells in the anterior chamber of the eye[3,4]. Fas is a type I transmembrane protein in the tumor necrosis factor receptor/nerve growth factor receptor family[1,2] and is expressed on a variety of cell types, including hepatocytes, activated B and T cells, and neutrophils[5]. Loss of function mutations in the Fas/FasL system result in lymphoproliferation and autoimmunity in both humans[6,7] and mice[8], demonstrating a critical role of this system in T- and B-cell regulation.

The immune privilege of tissues such as the testis[9] and the anterior chamber of the eye[3], has been attributed to local expression of Fas ligand, which presumably acts by inducing apoptosis of invading, Fas-bearing activated T cells. In addition, FasL expression on a subset of tumors may contribute to evasion of immune surveillance[10,11]. A provocative series of reports has suggested that FasL can prevent allograft rejection. For example, the acceptance of transplanted Sertoli cells across MHC barriers in a mouse transplantation model was attributed to FasL expression on those cells[9]. Recently, Lau and co-workers reported that syngeneic myoblasts engineered to express FasL could delay rejection of pancreatic islet allografts when cotransplanted under the kidney capsule[12].

Although the cotransplantation of islets and FasL-expressing myoblasts under the kidney capsule might appear to be an attractive method for improving clinical islet transplantation, islet transplantation under the kidney capsule is not effective in large animals[13,14]. Rather, islet engraftment is best achieved in large species by infusion into the portal vein with subsequent dispersion throughout the hepatic vasculature[14,15]. After dispersion in the hepatic vasculature, however, myoblast protection of islet allografts may be compromised by a failure of islet cells and myoblasts to colocalize. To circumvent this potential problem, we investigated whether direct FasL expression on transplanted islets could prevent allograft rejection.

We expressed FasL on islets by two distinct methods. First, we transduced murine islets *in vitro* with a replication-defective adenoviral vector expressing a murine FasL cDNA and implanted these islets below the kidney capsule of allogenic mice. As an alternative means of generating FasL-bearing islet cells, we generated transgenic mice that express FasL specifically on islet β cells. Here we report that FasL expression on islets does not confer immune privilege, but instead targets islets for rapid neutrophilic destruction.

Efficient adenoviral transduction of FasL on pancreatic islets

Recombinant adenoviral vectors effectively mediate gene transfer in intact pancreatic islets[14,16]. To assess the efficacy and tissue distribution of adenoviral gene transfer in our experimental system, iso-

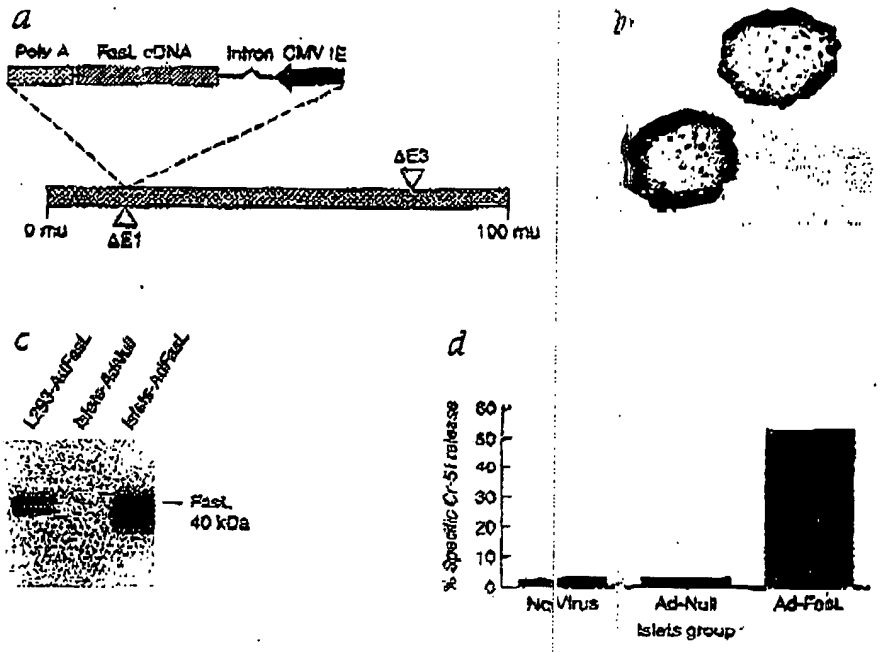


Fig. 1 Expression of functional Fas ligand (FasL) on explanted islets. *a*, The AdFasL vector carries a murine FasL cDNA under the control of the human cytomegalovirus intermediate early promoter (CMV IE). The polyadenylation signal [poly(A)⁺] is taken from simian virus 40. The vector has deletions in both the E1 and E3 regions of the adenovirus genome. *b*, Histologic sections of X-gal-stained islets 24 h after infection with an adenoviral vector expressing a nuclear targeted β-galactosidase. *c*, Western blot analysis of FasL expression. Immunoreactive FasL is detected in lysates from both 293 cells and islets transduced with AdFasL but not in islets infected with AdNull. *d*, Killing of Fas-bearing targets by AdFasL-transduced cells. ^{51}Cr-labeled, W4 cells (expressing Fas) were mixed with mock-infected islets (no virus), or islets infected with AdNull or AdFasL.

lated mouse islets were transduced with a vector encoding a nuclear targeted β-galactosidase reporter gene (AdRSVnLacZ), stained with X-gal, and embedded in plastic. Examination of histologic sections of the transduced islets revealed β-gal expression solely in the outermost one or two layers of islet cells (Fig. 1*b*), suggesting that the vector does not completely penetrate intact islets. To direct FasL expression on the islets, we generated a replication-defective adenoviral vector containing the murine FasL cDNA (ref. 17) under the control of the cytomegalovirus immediate-early promoter (AdFasL, Fig. 1*a*). The AdFasL vector directed expression of FasL in islets after infection *in vitro*, as assessed by western blot analysis (Fig. 1*c*). Transduction with AdFasL had minimal toxicity to islets for at least 7 days after gene delivery, as judged by light microscopy and by vital staining of cultured islets with dithizone[16]. AdFasL-infected islets specifically killed Fas-expressing W4 lymphoma cells (Fig. 1*d*)[17].

FasL expression accelerates rejection of islet grafts

We assessed whether FasL expression could confer immune privilege and thereby prolong survival of islet allografts. Islets from C3H mice ($H\text{-}2^k$) were transduced with AdFasL and, after a 24-hour incubation *in vitro*, transplanted under the kidney capsule of streptozocin-induced diabetic B6 mice ($H\text{-}2^b$). As controls, islets from C3H mice transduced with an adenoviral vector lacking the FasL expression cassette (AdNull) were transplanted into B6 mice. AdNull-infected islets were rejected in a mean of 9 days (Fig. 2*a*), which is similar to the rejection time for noninfected islets (data not shown). Strikingly, however, FasL-transduced islets underwent accelerated rejection (mean 4 days, $P < 0.001$). This accelerated rejection was not site-specific, as islets expressing FasL also underwent accelerated rejection when infused into the portal vein (data not shown). To investigate the possibility that the accelerated rejection was strain-specific (that is, C3H to

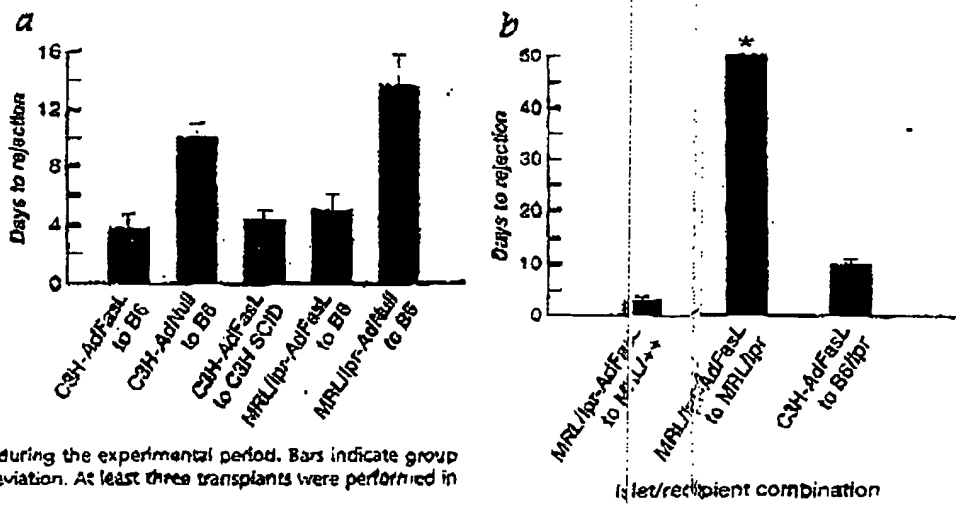


Fig. 2 *a*, Effect of islet FasL expression on graft survival. Experimental groups are listed using the mouse strain used as islet donor, followed by the adenoviral vector used to transduce islets, followed by the recipient strain. All grafts expressing FasL underwent accelerated rejection. *b*, Accelerated islet rejection requires host Fas expression. *No animals in the MRL/lpr-AdFasL to MRL/lpr group underwent graft rejection during the experimental period. Bars indicate group means; error bars indicate one standard deviation. At least three transplants were performed in each group.

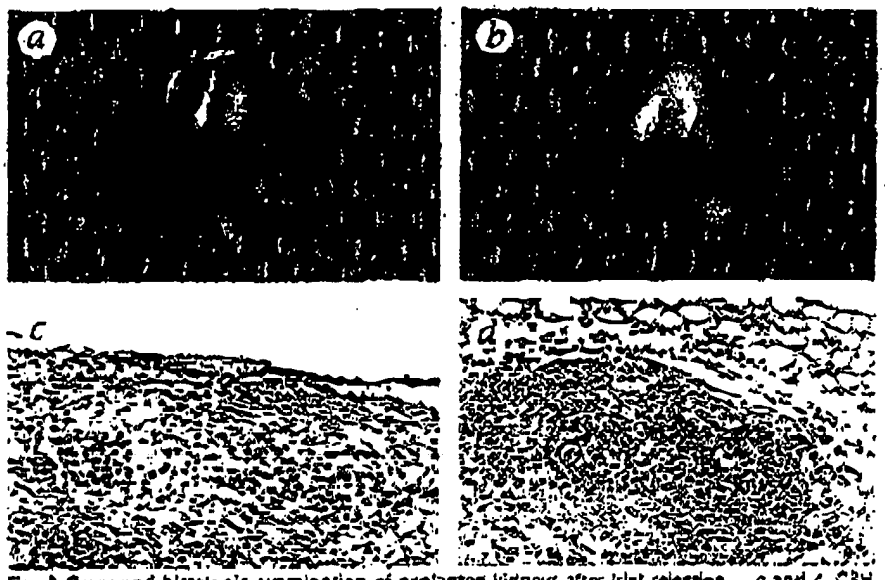


Fig. 3 Gross and histologic examination of explanted kidneys after islet rejection. *a* and *c*, C3H islets infected with AdNull and transplanted into B6 mice, examined 9 days after islet implantation. *b* and *d*, C3H islets infected with AdFasL and transplanted into B6, examined 4 days after islet implantation. Sections in *c* and *d* were stained with esterase; neutrophils stain red.

B6), we tested other combinations of donor and recipient mice. In all combinations tested (including BALB/c (H-2^d) to B6, DBA/2 (H-2^d) to B6, and BALB/c to DBA/2) rejection of FasL-expressing islets was accelerated.

FasL-mediated rejection is T- and B-cell independent

Islet allografts and xenografts survive indefinitely in severe combined immunodeficient (SCID) mice[17], which are deficient in lymphocytes of both the T and B lineages. To assess the role of T and B cells in the accelerated rejection of AdFasL-transduced islets, C3H islets infected with AdFasL were transplanted into diabetic C3H-SCID mice. The islets were rejected in a mean of 4 days, equivalent to the rejection time in fully immunocompetent B6 hosts (Fig. 2*a*).

Islet Fas expression is not required

Normal islets do not express Fas (ref. 20, 21). However, dispersed β cells can be induced to express Fas *in vitro* by interleukin-1β (IL-1β)[18]. In addition, Fas upregulation on β cells *in vivo* has been recently demonstrated in the spontaneous diabetic NOD model[19]. Therefore, the FasL-expressing islet grafts might have failed owing to self-destruction of β cells, mediated via upregulated Fas expression in islet cells after transplantation. To address this possibility, islets from MRL/lpr mice (H-2^k), which have a disruption in the *fas* gene[13,18], were transduced with AdFasL and transplanted into diabetic B6 hosts. These islets were rejected as rapidly as AdFasL-infected C3H islets (Fig. 2*a*). Thus, the destruction of FasL-expressing islets is not caused by Fas-mediated apoptosis of β cells.

Host Fas expression is required for accelerated rejection

To investigate whether accelerated rejection of FasL-expressing islets was dependent on Fas expression on host cells, we transplanted FasL-expressing islets into Fas-deficient *lpr* hosts. MRL/lpr islets infected with AdFasL were accepted indefinitely in MRL/lpr hosts (Fig. 2*b*). In contrast, MRL/lpr islets infected with AdFasL were rejected rapidly in congenic MRL/+ hosts, which carry a wild-type *fas* gene (mean 3 days, Fig. 2*b*). Thus, rapid destruction of congenic islets occurs by a Fas-dependent pathway. C3H islets infected with AdFasL were rejected in a mean of 10 days when transplanted into B6/lpr hosts, similar to the rejection time of C3H islets infected with AdNull transplanted into B6 hosts (mean 9 days, Fig. 2, *a* and *b*). Thus, accelerated rejection of both allogeneic and syngeneic islet grafts expressing FasL is dependent on Fas expression on host cells.

FasL expression induces a rapid neutrophilic infiltrate

On gross examination, rejecting islets of AdNull-islet/Fas$^+$ host and AdFasL-islet/Fas$^-$ host combinations appeared mildly hemorrhagic and inflamed, which is typical of islet allograft rejection (Fig. 3*a*). In contrast, rejecting islets in the AdFasL-islet/Fas$^+$ host combinations had a white, raised appearance, resembling abscess formation (Fig. 3*b*). As expected, histologic examination of explanted kidneys undergoing normal islet allograft rejection showed a lymphocytic infiltrate, with few cells staining positive for granulocyte-specific esterase[20] (Fig. 3*c*). Histologic examination of kidneys explanted from Fas$^+$ hosts after accelerated rejection of AdFasL-infected islets revealed a dense neutrophilic infiltrate. Esterase staining confirmed a neutrophil-predominant infiltrate (Fig. 3*d*). Neutrophilic infiltration was not a late result

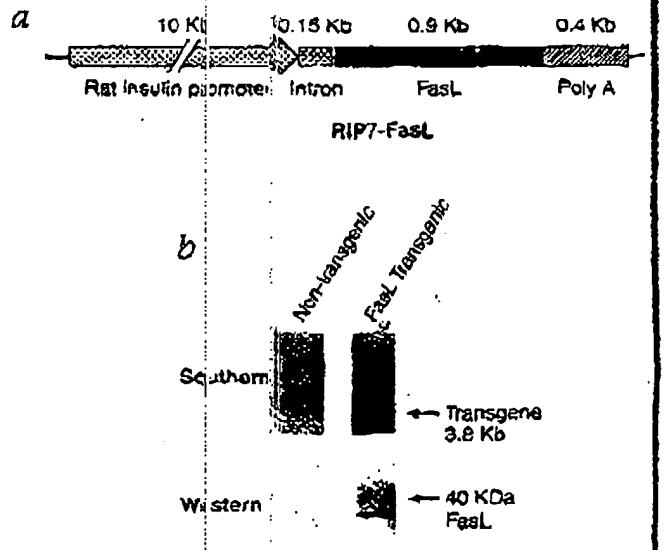
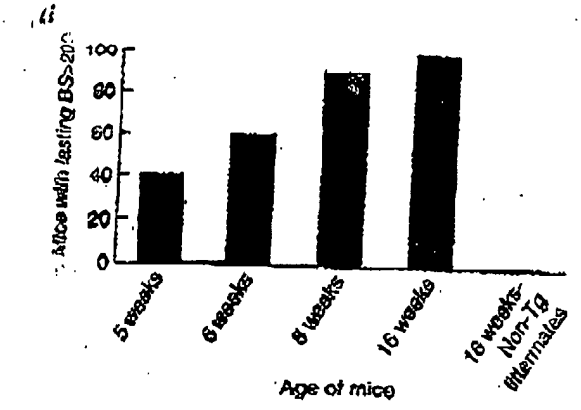


Fig. 4 RIP7-FasL transgenic mice. a, Construct used to generate FasL-transgenic mice. A murine FasL cDNA was spliced to a 10-kb fragment of the rat insulin promoter/enhancer, including the first insulin intron. The polyadenylation signal, poly (A)$^+$, was from the MHC class II E-α gene. b, Southern and western blot analysis of transgenic mice and nontransgenic littermates. Genomic DNA was digested with *Eco*RI and probed with the FasL cDNA. Islets were isolated as described in the Methods section before western blotting.



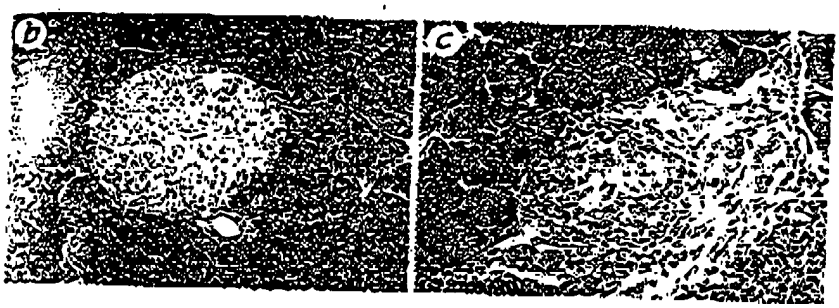


Fig. 5 Diabetes and neutrophilic infiltration of pancreatic islets in transgenic mice with islet cell-specific expression of FasL. *a*, The percentage of transgenic mice with fasting blood sugars (BS) above 200 mg/dl increased steadily over time. Nontransgenic littermates did not develop diabetes. *b*, Esterase stain of pancreas of a nontransgenic mouse at 16 weeks. *c*, Esterase stain of pancreas from a transgenic mouse at 6 weeks.

of islet necrosis because neutrophilic infiltrates were seen as early as 24 h after transplantation in the FasL-expressing grafts, when the graft was still functioning and the islet cells appeared viable (data not shown).

Transgenic mice expressing FasL on pancreatic β cells

To study the effect of FasL expression on islets in the absence of possible artifacts resulting from islet isolation, adenovirus-mediated gene transfer, or surgical trauma during transplantation, we generated transgenic mice expressing FasL specifically on pancreatic β cells. The transgenic construct expresses a murine FasL cDNA under the control of a rat insulin promoter/enhancer[24] (RIP7-FasL; Fig 4*a*). The RIP7 vector was chosen because it directs high levels of expression in β cells, with excellent fidelity[25]. A transgenic mouse line was generated (RIP7-FasL) containing eight copies of the RIP7-FasL construct. FasL protein expression was demonstrated by western blot analysis of isolated islets (Fig. 4*b*). FasL expression in the RIP7-FasL islets was one-tenth the level expressed on AdFasL-transduced islets, as determined by densitometry.

RIP7-FasL mice develop diabetes and neutrophilic infiltrates in islets

The RIP7-FasL transgenic mice developed diabetes at a young age; 0% of mice were diabetic by 5–6 weeks of age, and 100% of mice were diabetic by 16 weeks (Fig. 5*a*). Histologic examination of pancreata at 5 weeks of age revealed massive infiltration of neutrophils into islets (Fig. 5, *b* and *c*), and older transgenic mice showed progressive scarring and atrophy of islets (data not shown). Thus, germline expression of FasL in pancreatic β cells results in massive neutrophil infiltration, islet destruction, and diabetes.

Discussion

The major findings of this study are as follows: (1) FasL expression on islets of Langerhans results in neutrophilic infiltration and destruction of islets. (2) The mechanism of this accelerated rejection differs from that of alloimmune rejection in that it was T- and B-cell independent. (3) Accelerated rejection of FasL-expressing islets cannot be attributed to Fas-mediated apoptosis of islet cells, because Fas-deficient lpr islets (expressing FasL) underwent a similarly accelerated rejection. (4) Accelerated rejection of FasL-expressing islets was dependent on Fas expression in host tissues.

One possible explanation for our results is that high levels of FasL expression in islet cells results in nonspecific cell dysfunction and death, as reported in association with overexpression of other transgenes in pancreatic β cells, including H-ras and MHC class II antigens[26–28]. If islet cell death resulted merely from overexpression of FasL, the observed neutrophilic infiltration could be a secondary phenomenon. This explanation is, however, inconsistent with both past and present experimental data. Neutrophilic infiltration of islets was not associated with islet dysfunction and destruction in mice overexpressing MHC class II antigen and H-ras (refs. 26–28). Moreover, if islet cell death was a direct result of FasL overexpression, it is unclear why (in the present study) islet rejection occurred only in the presence of Fas expression by host cells.

Recently, Chervonsky *et al.*, showed that Fas expression on β cells can be induced *in vivo* in response to an adoptive transfer of diabetogenic T lymphocytes into 12-week-old NOD mice[21], raising the possibility that FasL expression on islet cells can result in self-induced apoptosis, and perhaps a subsequent neutrophilic infiltration as observed in our experimental system. However, Fas expression in the islet β cells does not appear to be involved in the islet destruction we observed. First, in the C3H/B6 background in which the transgenic mice were generated, a spontaneous infiltration of T cells into the islets of Langerhans is not observed. Thus it is unlikely that Fas expression is induced by T cells as an initiating event in the neutrophilic β-cell destruction found in the FasL transgenic mice. Furthermore, accelerated destruction of islet grafts expressing FasL occurred in SCID mice, which lack T lymphocytes, demonstrating that signals from T lymphocytes are not required for the granulocyte infiltration and islet cell destruction. Second, both lpr islets and wild-type islets (without the *lpr* mutation) were destroyed at similar rates, demonstrating that impairment of Fas expression in islets did not protect them from destruction. Since the *lpr* mutation, caused by the insertion of a transposable element in the *fas* gene, has been demonstrated to permit a low level of Fas expression[29,30], it could be argued that a low level of Fas expression on the lpr islets may lead to self-killing of FasL transduced islets. However, transplantation of FasL-expressing islets containing the wild-type *fas* gene into Fas-deficient lpr hosts completely eliminated the neutrophilic destruction, demonstrating a requirement for Fas expression on host cells rather than on islet cells. We there-

fore conclude that the destruction of pancreatic islet cells observed in our study is not due to an upregulation of Fas on pancreatic β cells and an autocrine Fas–FasL-mediated suicide.

Our data are most consistent with the hypothesis that FasL expression results in the recruitment of neutrophils into islet tissue, leading to islet destruction. This hypothesis is supported by the data of Seino *et al.*, which showed that tumor lines stably transfected with FasL undergo neutrophil-dependent destruction after subcutaneous implantation[29]. We have similarly found rapid neutrophilic infiltration of several FasL-expressing cell lines implanted under the kidney capsule (data not shown). Lau and co-workers[12] did not observe a neutrophilic response to FasL expressing myoblasts, which in their study prolonged islet allograft survival. In contrast, we have found that syngeneic myoblasts expressing FasL undergo rapid neutrophilic destruction when implanted under the kidney capsule (S.-M.Kang, A. Hofmann, D. Lee, P.G. Stock, H. Blau, manuscript in preparation). Moreover, coimplantation of FasL-expressing myoblasts with unmanipulated allogeneic islets accelerated rejection. Further investigations will be required to resolve these divergent findings.

Our data also indicate that neutrophil recruitment is dependent on islet expression of FasL and host expression of Fas. However, the precise molecular mechanism of neutrophil recruitment in this setting remains to be elucidated, in particular, the identification of the cell type in which Fas expression is critical for the phenotype. This mechanism is of substantial interest because abrogation of neutrophil recruitment might unmask the ability of FasL to protect allografts from T cell-mediated rejection. In this regard, FasL mediates upregulation of IL-8 expression in a colon carcinoma cell line, HT-29 (ref. 30), and in synoviocytes[31]. IL-8 is critical for neutrophil chemotaxis and activation[32]. It is therefore possible that expression of FasL results in upregulation of IL-8 expression in surrounding tissues, leading to recruitment of neutrophils[33,34]. However, it is also possible that soluble FasL, which is released from the cell surface by matrix metalloprotease cleavage of membrane-bound FasL (ref. 34–36), can act directly as a neutrophil chemotactic factor. Consistent with this hypothesis, Fas is highly expressed on neutrophils[37,38].

If FasL expression results in neutrophil chemotaxis, by what mechanism do FasL-expressing tissues such as the eye[4] and the testis[5] escape neutrophil-mediated destruction? One possibility is that these sites are intrinsically less susceptible to neutrophil chemotaxis, perhaps because of low or absent IL-8 production in response to FasL. A second possibility is that the level of FasL expression at these sites is below a threshold at which stimuli for neutrophil infiltration are generated. Relevant to this possibility, we have recently generated a second transgenic mouse line that expresses approximately one-fourth the levels of FasL on β cells as compared with the RIP7-FasL line described above. This second line of mice expresses functional FasL on β cells, as judged by islet cytotoxicity to W4 cells; however, the mice do not develop neutrophilic islet infiltrates or diabetes. In preliminary transplantation experiments, the survival of islets grafted from this line of transgenic mice was similar to survival of islet grafts from nontransgenic littermates (data not shown). Thus, the level of FasL expression on cells of these mice appears insufficient to cause neutrophil accumulation, but also does not confer immune privilege. Independently, Allison *et al.* have generated a FasL transgenic mouse line using a shorter version of the rat insulin promoter[39]. These mice also develop neutrophilic infiltrates in the islets, but do not develop diabetes. Survival of islet transplants from these mice are neither shortened nor prolonged compared with islet transplants from nontransgenic littermates. Thus, they appear to have a phenotype intermediate between those seen in our two lines, which may suggest a dose-response relationship between FasL expression levels and the intensity of neutrophilic infiltration. However, FasL-mediated protection was not seen in any of the mouse lines. A third potential explanation for the lack of neutrophilic infiltrates in the eye and testis is low or absent expression of the matrix metalloprotease(s) that cleave FasL to soluble FasL (ref. 34–36). In the absence of cleavage and release of soluble FasL, the action of FasL would be limited to cells in direct contact with FasL-expressing cells.

Our findings are relevant to the development of FasL-based strategies to modify immune responses. Although other studies suggest a role of FasL in preventing allograft rejection[4,5], we have found in a clinically relevant transplant model that FasL acts as a proinflammatory factor that accelerates, rather than prevents, graft loss. This proinflammatory function of FasL will require further characterization and modulation before FasL-based immunotherapies can be applied.

Methods

Adenoviral vectors. AdFasL: The 940-bp coding region of the murine FasL cDNA (ref. 17; kindly provided by S. Nagata) was excised from pBL-MFLwg by *Xba*I digestion, and was ligated into the *Xba*I site of pCI (Promega, Madison, WI), which contains the CMV immediate-early enhancer/promoter, a chimeric intron, and the SV-40 polyadenylation signal (Fig. 1*a*). A *Fspl/Bam*HI fragment containing the pCI-FasL expression cassette was then cloned into *Bam*HI/*Eco*RV-restricted pΔE1sp1A (Microbix Biosystems, Toronto, Ontario; a left-end adenovirus shuttle plasmid containing Ad5 sequences from map units 0 to 15.8 and a 3.2 kb E1 deletion) to generate pΔE1sp1A-FasL. AdFasL was generated by homologous recombination in 293 cells following cotransfection of linearized pΔE1sp1A-FasL and the 33 kb *Cla*I fragment of Ad-dl327 (an E3-deleted Ad)[40]. AdRSVnLacZ: The construction of AdRSVnLacZ, an E1- and E3-deleted recombinant adenovirus vector containing a nuclear targeted *Escherichia coli* β-galactosidase gene under the control of the RSV long terminal repeat promoter, has been described in detail previously[41]. AdNull: This virus contains the E1 and E3 deletions identical to those in AdFasL, but it does not contain a transgene. AdNull was generated by homologous recombination between the 33-kb *Cla*I fragment of Ad-dl327 and linearized pΔE1sp1A. All vectors were amplified in 293 cells, purified, and titered as described[40]. Viral stocks were free of replication-competent virus as determined by assays capable of detecting replication-competent virions at a frequency of 1 in 10^7.

Cytotoxicity assay. Fifty islets were mixed with 1×10^4 Fas-expressing ^{51}Cr-labeled W4 cells[17] (kindly provided by S. Nagata) in a volume of 200 μl in wells of a 96-well V-bottom plate. After overnight incubation, radioactivity in the supernatant was measured by scintillation counting (Wallac, Gaithersburg, Maryland). Specific killing was calculated as [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. All groups were run in triplicate.

Transplants. Murine islets were isolated by collagenase digestion and purification on dextran gradients, followed by hand-picking, as described[42]. Purified islets were cultured for 2 h in islet medium [10% FCS in RPMI (Life Technologies, Grand Island, NY)] before infection. Islets were then transferred to reduced serum medium (2% FCS in RPMI). Adenovirus (AdNull or AdFasL) was added at a multiplicity of infection of 2.25, with the assumption that each islet contains approximately 1×10^3 cells. After a 2-h incubation, the islets were transferred into islet medium and cultured for an additional 20–24 h before transplantation. Diabetes (defined as at least two consecutive days with blood glucose >350 mg/dl) was induced in recipient mice by intraperitoneal injection of 300 mg/kg of streptozocin. Each recipient received 800–1000 islets under the left renal capsule via a 27 gauge needle. Rejection was defined as blood sugars >250 mg/dl on two consecutive days, following at least one post-transplant glucose <150 mg/dl. A min-

immunofluorescence of three independent transplants was performed for each islet/host combination.

Western blot. Samples were lysed in RIPA buffer (PBS with 1% vol/vol NP-40, 0.5% wt/vol sodium deoxycholate, 0.1% wt/vol SDS), and were resolved on a 12% (wt/vol) SDS polyacrylamide gel. Duplicate gels were run and stained with Coomassie blue to confirm approximately equivalent protein loading between samples. Proteins were transferred to nitrocellulose. After blocking with 20% nonfat dry milk in Tris-buffered saline with 0.1% (vol/vol) Tween 20, FasL protein was detected with a polyclonal rabbit anti-FasL antibody (N-20, Santa Cruz Biotechnology, Santa Cruz, CA), per the manufacturer's instructions. Bound antibody was detected with peroxidase-coupled anti-rabbit IgG and enhanced chemiluminescence (ECL: Amersham, Arlington Heights, IL). A densitometer (Bio-Rad, Hercules, CA), was used to compare signal intensity.

Transgenic mice. The murine FasL cDNA was adapted with *Cla*I ends via polymerase chain reaction (PCR), using the primers gtCGGGATCGATCGATAAGGAAACCCTTTCCTGG and dCCCAATTCATCGATTCCTGGTGCCCATGATAAAG. The PCR product was ligated into the *Cla*I site of the RIP7 vector[29]. After the FasL cDNA was sequenced, the construct was linearized and injected into C3H/B6 F, embryos. Transgenic mice were identified by Southern blot analysis, and copy number was determined by densitometry. Mice were bred into the C3H background for one to two generations before analysis.

Histology. Explanted tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and processed for hematoxylin and eosin staining or a granulocyte-specific stain that employs naphthol AS-D-chloroacetate as a substrate for esterase[30]. Granulocytes appear red with the esterase stain. Expression of β-galactosidase in intact islets fixed in 2% formaldehyde/0.2% glutaraldehyde was detected with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal). X-gal-stained islets were embedded in plastic, cut in 5-μm-thick sections, and counterstained with nuclear fast red.

Statistics. Results are graphed as the mean ± 1 s.d. Differences were assessed using unpaired Student's t-test with the aid of SigmaStat software (Jandel Scientific, San Rafael, CA).

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